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13. ABSTRACT (Maximum 200 Words)

Receptors for vasoactive intestinal peptide (VIP-R) are overexpressed in human breast cancer. This phenomenon may have important diagnostic and therapeutic implications because carrier systems such as sterically stabilized liposomes (SSL) loaded with imaging or therapeutic agents, and with surface ligands specific to VIP-R could potentially be actively targeted to breast cancer. This part of the project aims to test the targeting ability of VIP–SSL to n-methyl nitrosourea (MNU)-induced rat breast cancer in vitro. First, VIP was conjugated to an activated DSPE-PEG (DSPE–PEG –NHS) under mild conditions to obtain a predominantly 1:1 conjugate of VIP and DSPE–PEG (DSPE–PEG –VIP), as evidenced by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). To test breast cancer targeting ability in vitro, DSPE–PEG –VIP was inserted into preformed fluorescent cholesterol (BODIPY-Chol) labeled SSL by incubation at 37°C. These VIP–SSL were incubated with MNU-induced rat breast cancer tissue sections. The results showed that when compared to fluorescent SSL without VIP or non-covalently attached VIP, significantly more VIP–SSL were attached to rat breast cancer tissues indicating that SSL with covalently attached VIP can be actively targeted to rat breast cancer tissues. This targeted carrier system is currently being explored for functional imaging of breast cancer.

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VIP-R as molecular target for breast cancer

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INTRODUCTION

The primary goal of the past year's work was to prepare and characterize the targeted sterically stabilized liposomes incorporating Tc99m inside and vasoactive intestinal peptide (VIP) on the surface. This targeted system would then be tested in vitro using rat breast cancer tissues, for its binding to breast cancer to find if it is improved in the presence of VIP

BODY

TASK 1: Develop labeled VIP-SSL

a. **Prepare sterically stabilized liposomes (SSL) with average size of 100 nm**
b. **Label SSL by trapping Tc99m-HMPAO irreversible in the liposomes**
c. **Conjugate VIP to DSPE-PEG-NHS**
d. **Insert DSPE-PEG-VIP on the surface of labeled SSL**
e. **Determine the size, phospholipid and VIP contents, labeling efficiency of the final liposomes product**
f. **Optimize the final composition and procedure**

Preparation of SSL:

Sterically stabilized liposomes were prepared by hydration of dried lipid film followed by extrusion, as described before (Dagar, 1998) with modifications. Egg-phosphatidylcholine (PC), cholesterol (CH), polyethylene glycol (molecular weight 2000) conjugated distearyl phosphatidylethanolamine (DSPE-PEG) & dipalmitoyl phosphatidylglycerol (DPPG) in the molar ratio PC: DPPG: DSPE-PEG: CH of 0.50:0.10:0.05:0.35 were used to form the sterically stabilized liposomes. The lipid mixture was dissolved in an organic solvent (chloroform-methanol; 9:1 v/v) & solvent evaporated in a rotary evaporator under vacuum. The dry lipid film was hydrated with isotonic, 50 mM glutathione containing isotonic 0.01M HEPES buffer (pH 7.4). The dispersion was extruded through polycarbonate filter (100 nm). The unentrapped glutathione was removed by gel filtration with isotonic 0.01M HEPES buffer (pH 7.4) as the eluent. The glutathione containing liposomes, visible as turbid fractions, were pooled and then labeled with Tc-99m-HMPAO as described below. The mean size of the prepared liposomes were measured using QuasiElastic Light Scattering.

Results: SSL with average size of ~100 nm were successfully prepared.

Labeling of SSL:

We adapted an efficient Tc-99m loading procedure for SSL from the literature (Boerman et al., 1997), with modifications (Dagar, 1998). The labeling was performed immediately after the free glutathione was removed by gel filtration in the preparation of liposomes. Briefly, Ceretec® was incubated with freshly eluted Tc99m-pertechnate to form a lipophilic Tc99m-HMPAO complex. This lipophilic complex was then incubated with preformed glutathione-containing liposomes and the complex, being lipophilic, passed through the bilayer. Tc-99m-HMPAO complex was then trapped irreversibly in the internal aqueous phase of the liposome by reduction of the lipophilic complex by

glutathione into a hydrophilic one. The free label was then removed by gel filtration. Fractions were collected and the radioactivity in each fraction measured using a dose calibrator. The Tc-99m-HMPAO encapsulating liposomes (turbid fractions with high radioactivity) coming out in the void volume were pooled and used for further studies. This method permitted a clear separation of the free Tc99m-HMPAO from the encapsulated Tc99m-HMPAO as seen in following Figure 1.

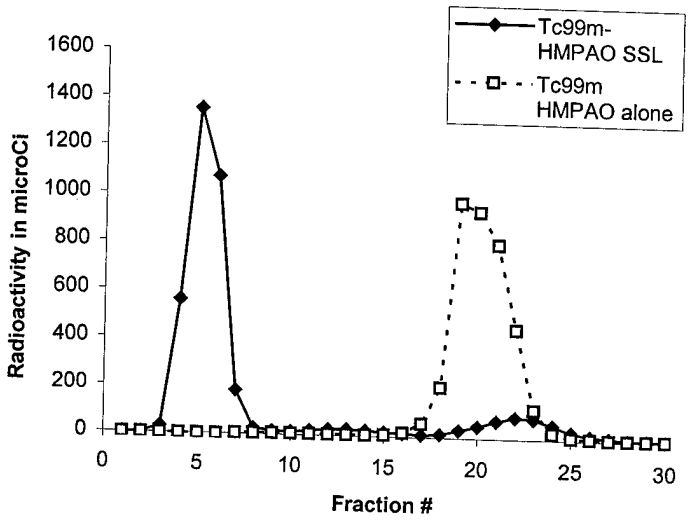

Figure 1: Elution profile of free Tc99m-HMPAO and Tc99m-HMPAO encapsulating SSL after gel filtration

Conjugation of VIP to DSPE–PEG:

An activated DSPE–PEG_{3400} (DSPE–PEG–NHS,1,2-dioleoyl-*sn*-glycero-3- 3400 phosphoethanolamine-*n*-[poly(ethylene glycol)]-*N* hydroxy succinamide, PEG *Mw* 3400) was used to conjugate VIP to DSPE–PEG . This 3400 reaction takes place between amines and NHS group, which acts as the linking agent. VIP and DSPE–PEG–NHS in the molar ratio of 1:5 (VIP: DSPE–PEG–NHS) were dissolved separately in 3400 0.01 M isotonic HEPES buffer, pH 6.6. DSPE–PEG–NHS solution was added in small increments over 1–2 min to the VIP solution at 48°C with gentle stirring. The reaction was allowed to proceed for 2 h at 48C and then stopped by adding glycine solution to the reaction mixture to consume the remaining NHS moieties. The conjugation was tested using SDS–PAGE and subsequent staining with first Coomassie Blue R-250 and then silver stain. The bioactivity of the conjugated VIP was tested using an in situ hamster cheek pouch bioassay. The VIP conjugated to DSPE–PEG (DSPE–PEG–VIP) was subsequently used to prepare fluorescent VIP–SSL.

Results: A 1:1 conjugate of DSPE-PEG and VIP was successfully prepared (Figure 2). In addition in situ bioassay indicated that the bioactivity of VIP was retained after conjugation.

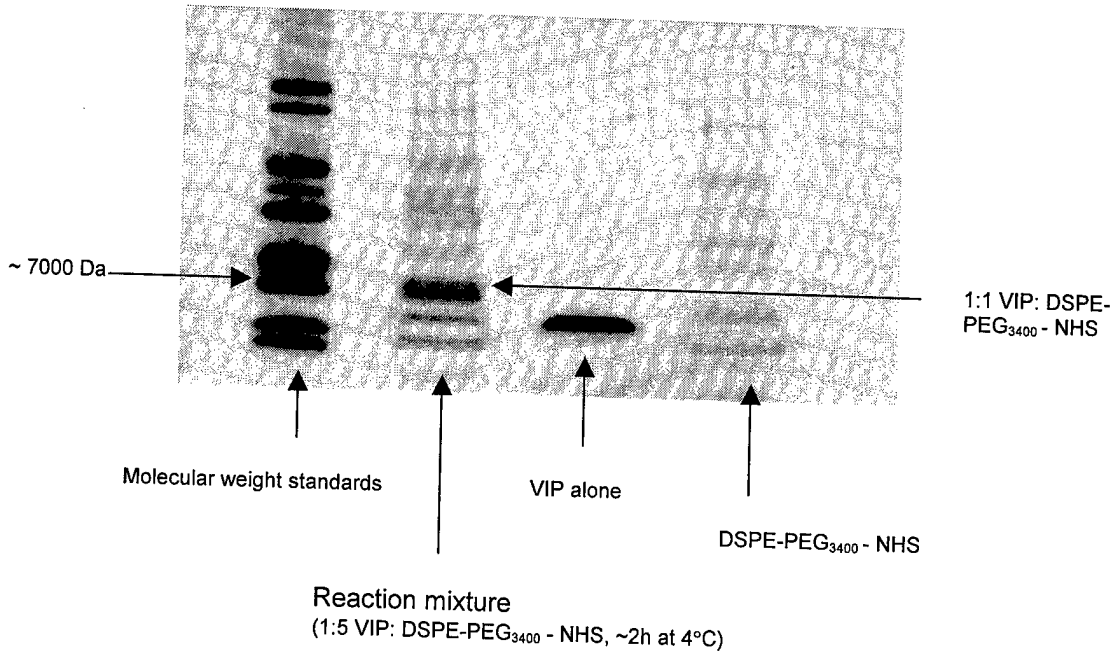

Figure 2: SDS-PAGE of the reaction products.

Insertion of DSPE-PEG-VIP into preformed SSL:

The conditions for DSPE-PEG conjugated VIP insertion into preformed Tc-99m-HMPAO encapsulating SSL were determined by measuring the amount of DSPE-PEG in the preformed liposomes under various conditions and times.

Results: These experiments indicated that DSPE-PEG was maximally inserted at about 2h. There was no significantly more insertion into the liposomes after 2h. Hence, 2h incubation at 37°C was considered enough to insert significant amounts of DSPE-PEG-VIP into the preformed SSL.

Stability of Tc99m-HMPAO encapsulating SSL at 37°C:

The insertion of DSPE-PEG_{3400} into preformed Tc-99m-HMPAO encapsulating SSL was done by incubation at 37°C. These conditions could cause leakage of encapsulated Tc-99m-HMPAO from the liposomes and could affect the size of the liposomes. Hence, the stability of Tc99m-HMPAO encapsulating SSL was tested by incubation of SSL in the presence and absence of DSPE-PEG_{3400}-NHS at 37°C.

Results: No significant leakage of Tc99m label (Figure 3) and change in size (Before incubation 109±13 nm and after incubation 114±15 nm) was observed, indicating that these liposomes were stable.

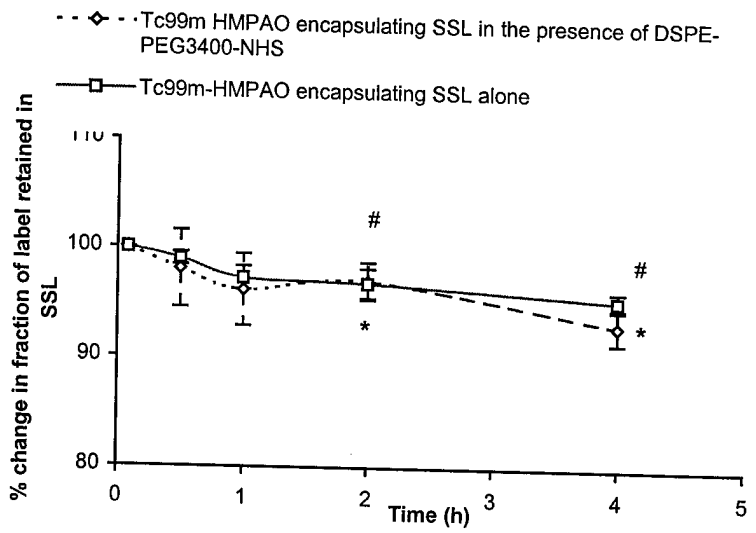

Figure 3: Leakage of encapsulated Tc99m-HMPAO from sterically stabilized liposomes (SSL) after incubation at 37°C in isotonic 0.01M HEPES buffer (pH 7.4). Each value is Mean ± SD, n = 3. * $p<0.05$ as compared to 0h (Tc99m-HMPAO encapsulating SSL in the presence of DSPE-PEG_{3400}-NHS). # $p<0.05$ as compared to 0h (Tc99m-HMPAO encapsulating SSL alone).

Characterization of Tc99m-HMPAO encapsulating VIP-SSL

The Tc99m-HMPAO encapsulating VIP-SSL were characterized in terms of their size, phospholipid and radioactivity content and their labeling efficiency and compared to Tc99m-HMPAO encapsulating SSL.

Results: There was no significant difference between Tc99m-HMPAO encapsulating VIP-SSL and SSL (Table 1) indicating that insertion of VIP did not interfere with the properties of the SSL.

Table 1: Characteristics of Tc99m-HMPAO encapsulating SSL and VIP-SSL (Each value is Mean ± standard deviation, n = at least 6)

CHARACTERISTIC	METHOD	Tc99m-HMPAO encapsulating SSL	Tc99m-HMPAO encapsulating VIP-SSL
SIZE	Qausi - elastic light scattering	109.81 ± 14.21 nm	114.77 ± 13.72 nm
PHOSPHOLIPID CONTENT	Modified Bartlet Phosphate assay	3.15 ± 0.23 µmol/mL	3.01 ± 0.48 µmol/mL
RADIOACTIVITY CONTENT	*Atomlab 100 dose Calibrator*	1008 ± 160 µCi/mL	800 ± 113 µCi/mL
LABELING EFFICIENCY	Atomlab 100 dose Calibrator	85.7 ± 4.46 %	83.8 ± 2.42 %

Status of Task 1: COMPLETED

Task 2: Test the in vitro Targeting of labeled VIP-SSL to VIP-R

a. **Develop breast cancer in rats with a carcinogen (MNU)**
b. **Prepare optimized labeled VIP-SSL containing small amounts of BODIPY-cholesterol**
c. **Determine the attachment of labeled VIP-SSL on sectioned rat breast cancer tissues using fluorescence microscope**
d. **Analyze and quantify the fluorescence images using Scion Image software.**

Breast cancer induction:

Breast cancer was induced in rats with MNU as previously described [Dagar 1998]. Briefly, virgin female Sprague–Dawley rats, 36 days old, weighing ~140 g, were anesthetized with ketamine/ xylazine (13.3 / 1.3 mg per 100 g body weight, i.m.). Each animal received a single intravenous injection of MNU (50 mg/kg body weight) in acidified saline (pH 5.0), via the tail vein. The rats were weighed weekly. They were palpated every week, starting at 3 weeks post-MNU administration. Palpable mammary tumors were detected within 100–150 days after injection.

Results: Breast cancer was successfully induced in female rats.

Preparation of fluorescent VIP–SSL:

For testing the in vitro binding, BODIPY-Chol (a non-exchangeable fluorescent probe) containing liposomes, were prepared with film rehydration-extrusion method, as described above but incorporating the probe at 1:1500 molar ratio (lipid:probe) in the lipid mixture. DSPE–PEG–VIP was inserted into these fluorescent liposomes to form fluorescent VIP conjugated sterically stabilized liposomes (VIP–SSL).

Results: Fluorescent VIP-SSL were successfully prepared

In vitro targeting of VIP–SSL to breast cancer:

The rats were euthanized by exposure to carbon dioxide in a closed chamber. Normal and cancerous breast tissues were excised, frozen immediately in liquid nitrogen and stored at 80°C until use. The frozen breast tissue was cut into 20 micron sections and mounted on microscopic slides. They were then fixed with 4% formaldehyde and allowed to air-dry for 10 min. Adjacent 5 micron thick frozen tissue sections, were stained with hemotoxylin and eosin to confirm the presence or absence of cancer in the breast tissue. The presence of VIP-R in these rat breast cancer tissues was confirmed using a fluorescent VIP, Fluo-VIP as described by us recently [Dagar 1999 and 2001]. Twenty-micrometer sections of MNU-induced rat breast cancer tissues were cut using a cryotome, placed on a slide, fixed with 4% formalin for 20 min, and then air-dried for 10 min. The BODIPY-Chol containing VIP–SSL were added to the sections and incubated for 1 h at room temperature. At the end of the incubation period, the slides were washed with 0.01 M isotonic HEPES buffer, pH 7.4, four times for 60s each. The slides were then observed with a Zeiss Fluorescence microscope attached to a Zeiss Camera (Carl Zeiss Inc., Thornwood, NY) and photographed. All photographs were taken with a 2-min exposure using Kodak Elite Chrome 400 photographic film.

Results: Figure 4 shows the fluorescence microphotographs of breast cancer tissues. The microphotographs indicate that more VIP-SSL were attached to MNU-induced rat breast cancer tissue sections while SSL without VIP or with non-covalently associated VIP, showed no significant attachment. This data indicated that VIP-SSL were able to bind to breast cancer tissue *in vitro* and it was likely that same would be true *in vivo*.

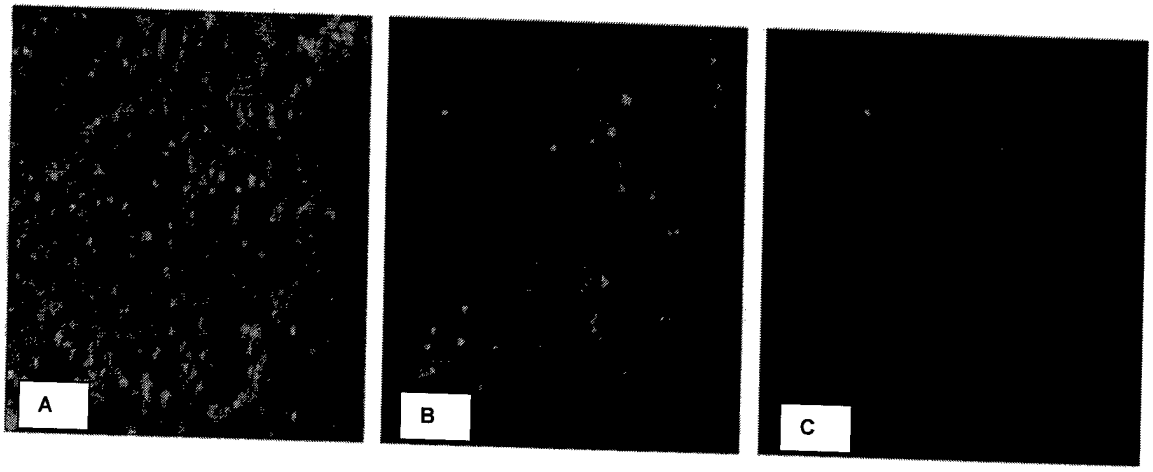

***Figure 4:** Microphotographs of MNU-induced rat breast tumor tissue sections incubated with fluorescent liposomes A. BODIPY-Chol incorporating fluorescent VIP-SSL (with covalently attached VIP) B. BODIPY-Chol incorporating fluorescent SSL (without VIP) C. BODIPY-Chol incorporating fluorescent SSL (with non-covalently associated VIP).

* We can provide electronic copies of Figure 4 in color if required.

Quantification of the fluorescence images:

We are in the process of evaluating and quantifying the fluorescent images obtained above using the Scion Image software.

Status of Task 2: COMPLETED except for part d.

KEY RESEARCH ACCOMPLISHMENTS

1. The Tc-99m-HMPAO labeled sterically stabilized liposomes were successfully prepared with a mean diameter of about 110 nm and a high Tc-99m labeling efficiency of about 85%..

2. Vasoactive intestinal peptide (VIP) was successfully conjugated to DSPE-PEG_{3400} at the N-terminal amine of the peptide. This DSPE-PEG_{3400} conjugated VIP retained the bioactivity of the native VIP.

3. The DSPE-PEG_{3400} conjugated VIP was used to form Tc-99m-HMPAO labeled sterically stabilized liposomes surface modified with VIP (VIP-SSL). No significant leakage of the encapsulated Tc-99m-HMPAO label and no significant change in size, phospholipid content and labeling efficiency were observed due to the insertion process. This method was simple and ensured that all the VIP molecules are on the outer surface of the liposomes available for interaction with the receptors.

4. The *in vitro* targeting studies using breast cancer tissues and VIP-SSL with a non-exchangeable label, fluorescent cholesterol (BODIPY-Chol) incorporated in the bilayer, confirmed the successful binding of VIP-SSL to rat breast cancer *in vitro*.

REPORTABLE OUTCOMES

Proceedings

Önyüksel, H., Dagar, S., Krishnadas, A., Blend, M., Rubinstein, I., "VIP-Liposomes for active, cell-specific targeted delivery to breast cancer in vivo", *NCI & CRS Second International Symposium on Tumor Targeted Delivery Systems*, Rockville, MD, 2002.

Manuscripts

Sumeet Dagar, Israel Rubinstein, Hayat Onyuksel, Liposomes in Ultrasound and Gamma Scintigraphic Imaging, *Methods in Enzymology*, 373, 2003 In Press.

Pending Grants (based on work supported in this grant)

National Institute of Health, PAR-03-124 Novel technologies for in vivo imaging (R21/R33), Hayat Onyuksel (PI), "In vivo Molecular Imaging of Breast Cancer".

National Institute of Health, PA-03-058 Exploratory/Developmental (R21) Bioengineering Research Grants, Hayat Onyuksel (PI), " Self Assembled Phospholipid Constructs for Targeted Drug Delivery to Breast Cancer.

National Institute of Health, PAR-03-045 Nanoscience and Nanotechnology in Biology and Medicine (R 21), Hayat Onyuksel (PI), "Breast Cancer Targeted Phospholipid Nanoparticles for Effective Chemotherapy".

CONCLUSIONS

We have successfully conjugated VIP to DSPE–PEG and incorporated this conjugated VIP into DSPE-PEG_{3400} preformed sterically stabilized liposomes to form a VIP–SSL construct. We have also shown the feasibility of this novel construct to actively target to MNU-induced rat breast cancer in vitro.

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APPENDICES

1. Dagar, S., Stastny, J., Blend, M., Rubinstein, I. And Onyuksel, H.: Preparation of Tc99m-HMPAO VIP-SSL for breast tumor detection. *Pharm.Sci.* 1:S-294, 1998
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INDUCTION OF ANTIGEN-SPECIFIC IMMUNE RESPONSES BY TRANSDERMAL DELIVERY OF ANTIGENS FORMULATED IN A NOVEL LIPID-BASED BIPHASIC DELIVERY SYSTEM.

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Purpose. The development of non-invasive methods for the delivery of proteins through the permeability barriers, such as the intact skin, will greatly facilitate the administration of human and veterinary vaccines. In the present study we used recombinant leukotoxin (Lkt) and hen egg lysozyme (HEL) as model antigens to investigate the ability of a novel lipid-based biphasic delivery system to deliver vaccine antigens by the transdermal route, and induce humoral and cellular responses in mice. **Methods.** Mice were immunized by the transdermal route with 50 μ g of Lkt or HEL formulated in a novel lipid-based biphasic delivery system. Control animals received patches containing formulation alone. The immunization was repeated on day 21 and the animals were euthanized 10 days later. **Results.** Transdermal delivery of a Lkt or HEL induced antigen-specific humoral and cellular responses. Both antigens induced antigen-specific antibody responses in serum that were primarily due to enhancement of IgG1 antibody subclass. The cellular response was characterized by the predominant induction of antigen specific IL-4 over IFN-γ. The proportion IL-4 secreting cells was higher in the draining lymph nodes than in the spleen of immunized mice. **Conclusions.** Our results indicated that topical application of antigens formulated in a novel lipid-based biphasic delivery system induced antigen-specific immune responses and demonstrated the feasibility of using this technology for the development of non-invasive methods of vaccine administration.

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PREPARATION OF STERICALLY STABILIZED VIP - LIPOSOMES ENCAPSULATING ^{99m}Tc-HMPAO FOR BREAST CANCER IMAGING.

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Purpose. Vasoactive Intestinal peptide receptors (VIP-R) are expressed in breast cancer. We have previously developed sterically stabilized liposomes (SSL) containing VIP associated with the lipid bilayer. The purpose of this study was to prepare VIP–SSL encapsulating ^{99m}Tc-HMPAO (^{99m}Tc-d,l-hexamethylpropylene amine oxime) for scintigraphic imaging of breast cancer. **Methods.** Liposomes encapsulating glutathione (GSH), were prepared by hydrating the dried lipid film composed of egg phosphotidylcholine, dipalmitoyl-phosphotidylglycerol, cholesterol and poly(ethylene)glycol (M.W. 2,000) conjugated to distearoyl-phosphotidylethanol-amine (molar ratio 0.50:0.10:0.35:0.05, respectively), using isotonic, HEPES buffer (pH 7.4), containing GSH. The mixture was extruded through polycarbonate membrane filters (100 nm and 50 nm) and free GSH was separated from liposomes by gel permeation chromatography (GPC). Liposomes were then labeled by incubation with lipophilic ^{99m}Tc-HMPAO complex, which is trapped, in the internal aqueous phase of liposomes by reduction with GSH. Unencapsulated ^{99m}Tc-HMPAO was removed by GPC. VIP was incorporated onto labeled liposomes by incubation at room temperature for 20 minutes. Mean size of labeled VIP–SSL was measured by quasi-elastic light scattering. Phospholipid and VIP concentrations were determined by phosphate assay and ELISA, respectively. Radioactive content of liposomes was, determined by Atomlab 100-dose calibrator. **Results.** Mean size of liposomes was 122± 6 nm (n=3). Phospholipid and VIP concentrations in liposomes were 23.75± 2.45 mM and 1.38± 0.21μ M, respectively (n=3). Labeling efficiency was 47.6± 2.5 % (n=3). **Conclusions.** Sterically stabilized, relatively small VIP liposomes were successfully labeled with ^{99m}Tc-HMPAO, suitable for breast cancer targeted imaging.

IDENTIFICATION AND TARGETING OF VIP RECEPTORS IN RATS WITH CHEMICALLY INDUCED BREAST CANCER

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INTRODUCTION

Targeted delivery to tumors exploits morphological and functional differences between cancerous and normal tissues. This can be used for selective delivery of anticancer drugs and imaging agents to tumors for therapy and early detection, respectively. Vasoactive intestinal peptide receptors (VIP-R) are expressed in human breast cancer in large numbers and with high affinity for VIP (1). Conceivably, methods that selectively target VIP-R could be advantageous in detecting and delivering anticancer drugs and imaging agents to breast cancer. To this end, we have developed sterically stabilized liposomes (SSL) with VIP associated on the surface (2). Rats bearing carcinogen (n-methyl nitrosourea [MNU])-induced breast cancer have been used to study the evolution of breast cancer (3). Whether VIP receptors are overexpressed in this cancer is uncertain. The objectives of this study were to determine the presence of VIP-R in MNU-induced breast cancer in rats, and to target VIP-R using VIP on SSL.

EXPERIMENTAL METHODS

Breast cancer induction

Breast cancer was induced in rats with MNU as previously described in the literature (4). Briefly, virgin female Sprague-Dawley rats, 36 days old, weighing ~140 g were anesthetized with ketamine/xylazine. Each animal received a single intravenous injection of MNU (50 mg/kg body weight) in acidified saline (pH 5.0). Rats developed palpable breast cancer within 60 – 90 days after injection.

Morphology

Normal and cancerous breast tissues were dissected from killed rats, frozen immediately in liquid nitrogen and stored at –70 °C until use. Tissue sections were stained with hemotoxylin & eosin and Fluo-VIP™ (Advanced Bioconcept). For the latter, sections were incubated with 40 nM Fluo-VIP™ for 2 h at room temperature. Thereafter, slides were washed with buffer and observed under a fluorescence microscope. Photographs were taken with a 2-min exposure. To determine specificity of Fluo-VIP™ binding, tissue sections were incubated with 40 nM Fluo-VIP™ followed by 1,000-fold excess unlabeled VIP.

Imaging

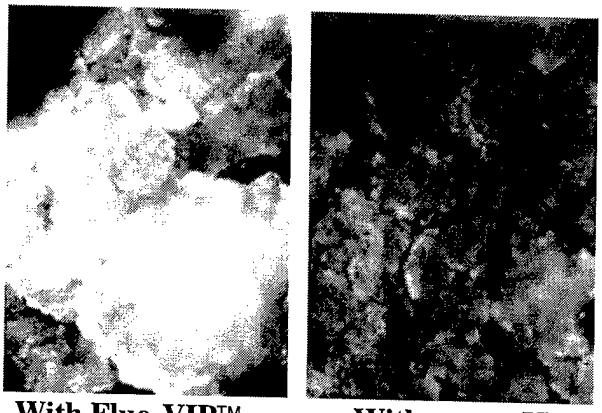
Tc99m-SSLs were prepared in the presence or absence of VIP as previously described (5). Rats were anesthetized with ketamine/xylazine and injected with ~500 μCi Tc99m-VIP SSL or Tc99m-SSL via the tail vein. Rats were placed prone on one head of a three-head gamma camera (Picker Prism 3000 XP). Planar images were acquired at 1 and 24 h post injection. Tc99m-VIP SSL was administered to rats with normal and cancerous breast while Tc99m-SSL was injected only to rats with breast cancer. Regions of interest (ROIs) were drawn to obtain counts per pixel.

RESULTS AND DISCUSSION

Fluo-VIP™ bound to VIP receptors in MNU-induced breast cancer. This binding was highly specific because fluorescence was significantly reduced in the presence of excess unlabeled VIP (Fig. 1). Moreover, binding of Fluo-VIP™ to normal breast was significantly less than that to cancerous breast and was further reduced in the presence of excess unlabeled VIP. Taken together, these data

suggest that MNU-induced breast cancer in rat is superior to a xenograft breast cancer model because it develops *in situ* and mimics human breast cancer (6). Similar to breast cancer in humans, this study documents overexpression of VIP-R in MNU-induced breast cancer in rats making this model suitable to study VIP-R targeting.

Figure 1. MNU-induced breast cancer.



With Fluo-VIP™ **With excess VIP**

Results of the imaging experiments showed significant targeting of Tc99m-SSL to breast cancer as compared to the normal breast (Table 1). However, there was no significant difference in targeting of Tc99m-SSL in the presence or absence of VIP. This may be related, in part, to dissociation of VIP from Tc99m-SSL at the receptor site.

Table 1

RATIO		*COUNTS/PIXEL*
Normal breast/background		0.81 ± 0.1
Tumor breast /background	VIP	2.06 ± 0.37*
	No VIP	3.04 ± 1.97^

Values are means±SEM; each group n=3. *p<0.05 in comparison to normal/background; ^p<0.05 in comparison to normal/background.

CONCLUSIONS

The results of this study indicate that MNU-induced breast cancer in rat overexpresses VIP receptors as compared to normal breast. They also show that Tc99m-SSL could be passively targeted to this cancer.

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Breast Cancer Research and Treatment **65:** 49–54, 2001.

Report

Detection of VIP receptors in MNU-induced breast cancer in rats: Implications for breast cancer targeting

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Key words: chemical carcinogenesis, Fluo-VIP, fluorescence microscopy, *N*-methyl nitrosourea, neuropeptides, PACAP, secretin, targeting, vasoactive intestinal peptide

Summary

Vasoactive intestinal peptide (VIP) is a 28 amino acid neuropeptide with a wide range of biological activities. Receptors for VIP (VIP-R) are overexpressed in breast cancer, where they may have diagnostic and therapeutic implications. Although *N*-methyl nitrosourea (MNU)-induced breast cancer in rats is used extensively as a model to study mammary carcinogenesis, there is no information about the expression of VIP-R in this model. Therefore, the purpose of this study was to investigate the presence of VIP-R in MNU-induced breast cancer in rats so that this model can be used to perform studies involving VIP-R. Breast cancer was induced in 36-day-old virgin female Sprague-Dawley rats, by a single intravenous injection of MNU (50 mg/kg body weight). The breast tumors were detected 100–150 days after injection. The normal and cancerous rat breast tissue were excised and 20 μ sections were incubated with 40 nM fluorescein-labeled VIP (Fluo-VIP^{TM}), in the presence and absence of 1000-fold excess unlabeled VIP, pituitary adenylate cyclase activating polypeptide (PACAP) or secretin. The sections were visualized under a fluorescence microscope and photographed. Fluo-VIP^{TM} stained rat breast cancer tissue homogeneously and to a much greater extent than normal rat breast tissue ($p < 0.05$). This staining was specific as indicated by displacement of Fluo-VIP^{TM} by excess unlabeled VIP and PACAP. Displacement of Fluo-VIP^{TM} by secretin indicated the probable presence of VIP receptors of type VPAC1 (VIP receptor subtype1) in the rat breast. These data suggest that, as in human breast cancer, VIP-R, predominantly of type VPAC1, are overexpressed in MNU-induced rat breast cancer tissue as compared to the normal rat breast tissue. Thus, MNU-induced rat breast cancer model can be used as a tool to study the functional role of VIP-R in human mammary carcinogenesis and VIP-R mediated active breast cancer targeting. This could have implications in the diagnosis, prognosis and therapy of human breast cancer.

Abbreviations: VIP: vasoactive intestinal peptide; VIP-R: VIP receptor; MNU: *N*-methyl nitrosourea; Fluo-VIP^{TM}: fluorescein labeled VIP; PACAP: pituitary adenylate cyclase activating polypeptide; VPAC1: VIP receptor subtype1; VPAC2: VIP receptor subtype2; SSL: sterically stabilized liposomes; H&E: hemotoxylin and eosin.

Introduction

Breast cancer is one of the leading types of cancer in women in the US, with approximately 43,000 women dying of breast cancer annually [1]. Vasoactive intestinal peptide (VIP) is a 28 amino acid amphipathic peptide initially isolated from porcine stomach [2]. It has a broad spectrum of neurotransmitter, neuroendocrine and immunomodulatory functions [3, 4]. There have been conflicting reports in the literature regarding the role of VIP in cancer. Some reports have shown that VIP stimulates proliferation of normal and malignant cells [5–7]. These reports suggest that VIP acts as a growth factor and the growth stimulation by VIP

could be in an autocrine fashion. On the other hand there have been reports in the literature, which suggest that VIP inhibits proliferation of cancer such as small-cell lung cancer [8]. The role of VIP in malignant disease remains unclear and more work is needed in this direction.

Functional VIP receptors (VIP-R) have been identified in various cancers such as prostatic cancer, colonic adenocarcinomas [9], pancreatic carcinoma [10], endometrial cancer, neuroblastoma and breast cancer [11]. *In vitro* studies using human breast cancer tissues and cells have shown the presence of high densities of VIP-receptors, with high affinity and specificity for VIP [11–13]. Even though breast cancers are frequently known to be polyclonal, studies have shown that VIP receptors exist homogeneously in surgically resected human breast tumors and biopsies, both primaries and metastases [11, 14]. High levels of VPAC1 receptor mRNA have been detected in human breast cancer cells and biopsy tissue samples, indicating that VIP receptor of type VPAC1 (VIP receptor subtype1) is present in human breast cancer [15, 16]. The presence of VIP-R in human breast cancer, may be important not only from prognostic point of view, but also may be relevant in use of VIP as an autologous targeting ligand of a delivery system. This could be advantageous in detection and therapy of breast cancer by active targeting of diagnostic agents and anticancer drugs to the breast cancer, after systemic administration.

To test VIP-R mediated targeting to breast cancer and to understand the role of VIP-R in breast carcinoma, *in vivo*, an animal model is required. Rats bearing carcinogen (*N*-methyl nitrosourea [MNU])-induced breast cancer have been extensively used to study carcinogenesis, evolution and chemoprevention of breast cancer [17–19]. This model is found to be similar to human breast cancer in many ways, including its metastatic ability, uniform morphology, uniform response to hormones, and being predominantly adenocarcinoma [17, 18]. Whether VIP-R are overexpressed in this breast cancer model is uncertain. The objective of this study was to determine if VIP-R are expressed in MNU-induced breast cancer in rats. Using histological sections of the breast and a fluorescein-labeled VIP (Fluo-VIP^{TM}), we have shown the overexpression of VIP-R in the MNU-induced breast cancer in rats. In our future studies we plan to use this animal model to study the targeting of imaging and therapeutic agents to VIP-R in order to advance the detection and therapy of breast cancer.

Materials and methods

Materials

Fluo-VIP^{TM} (fluorescein labeled VIP) was purchased from Advanced Bioconcept (Montreal, Quebec, Canada). VIP (human/rat) was synthesized, using solid-phase synthesis by Protein Research Laboratory at Research Resources Center, University of Illinois at Chicago. Rat PACAP (1–38) was obtained from American Peptide Co. (Sunnyvale, CA) and rat secretin from Peninsula Laboratories (San Carlos, CA). Tris–HCl, anhydrous 99% $MgCl_2$, bovine serum albumin (BSA) and bacitracin were obtained from Sigma Chemical Co. (St. Louis, MO). Virgin female Sprague Dawley rats (~ 140 g, age 36 days-old) were obtained from Harlan (Indianapolis, IN).

Methods

In conducting research using animals, the investigators adhered to the Institutional Animal Care Committee guidelines and to the *Guide for the Care and Use of Laboratory Animals*, prepared by Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Breast cancer induction

Virgin female Sprague Dawley rats were housed in controlled temperature and humidity, 12 h day and night cycle with chow and water given *ad libitum*. Breast cancer was induced with MNU as previously described in the literature with some modification [18]. Briefly, virgin female Sprague-Dawley rats, 36 days old, weighing ~ 140 g, were anesthetized with ketamine/xylazine (13.3/1.3 mg/100 g body weight, i.m.). Each animal received a single intravenous injection of MNU (50 mg/kg body weight) in acidified saline (pH 5.0), via the tail vein. The rats were weighed weekly. They were palpated every week, starting at three weeks post-MNU administration. Palpable mammary tumors were detected within 100–150 days after injection.

In vitro *binding of Fluo-VIP^{TM} to normal and cancer breast tissues*

The rats were euthanized by exposure to carbon dioxide in a closed chamber. Normal and cancerous breast tissues were excised, frozen immediately in liquid

nitrogen and stored at −70°C until use. The frozen breast tissue was cut into 20-μm sections and mounted on microscopic slides. They were then fixed with 4% formaldehyde and allowed to air-dry for 10 min. Adjacent 5 μm thick frozen tissue sections, were stained with hemotoxylin & eosin (H&E) to confirm the presence or absence of cancer in the breast tissue. Fluo-VIP™ solution was prepared in 50 mM Tris–HCl buffer, pH 7.4, 10 mM $MgCl_2$, 1% BSA and 1 mg/ml bacitracin was added to inhibit endogenous peptidases. The fixed 20 μ sections were incubated with 40 nM Fluo-VIP™ for 2 h at room temperature. Thereafter, slides were washed with 50 mM Tris–HCl buffer, pH 7.4, and 10 mM $MgCl_2$ four times for a minute each. They were then observed with a Zeiss Fluorescence microscope attached to a Zeiss Camera (Carl Zeiss Inc, Thornwood, NY) and photographed. All photographs were taken with a 2-min exposure using Kodak Elite Chrome 400 photographic film. To determine non-specific binding of Fluo-VIP™, the tissue sections, were incubated with 40 nM Fluo-VIP™ in the presence of 40 μM (1,000-fold excess) unlabeled VIP. The tissue sections were also incubated with 40 nM Fluo-VIP™ in the presence of 40 μM (1,000-fold excess) unlabeled secretin or PACAP.

The intensity of fluorescent staining of the tissue sections was graded by an independent reader not aware of the study design, on a scale of 0–4 with zero being the least and four being the most intense fluorescent staining (Table 1), as previously reported [20].

Table 1. Intensity of Fluo-VIP™ staining of rat breast tissues

	Fluo-VIP			
	Alone	With excess VIP	With excess PACAP	With excess secretin
Normal breast	1.25 ± 0.25	0.5 ± 0.29	0.50 ± 0.29	0.75 ± 0.25
Breast cancer	3.8 ± 0.20*	0.8 ± 0.20**	0.8 ± 0.20**	1.2 ± 0.20**

Values are means ± SEM, each group n = at least 4.
*$p < 0.05$ in comparison to Fluo-VIP™ alone on normal breast tissue.
**$p < 0.05$ in comparison to Fluo-VIP™ alone on breast cancer tissue.
Score of intensity of fluorescence (0 = minimum, 4 = maximum) observed in the breast tissue sections after treatment with Fluo-VIP™, in the presence and absence of unlabeled VIP, secretin and PACAP. Higher score indicating more staining and lower score indicating less staining.

Statistical analysis

Data are expressed as mean ± SEM. These scores were statistically analyzed using Kruskal-Wallis rank sum test and Wilcoxon test, to determine the differences in fluorescence intensity of different stained sections. A p-value < 0.05 was considered as statistically significant.

Results

Breast tissue specimens from at least four rats were used in these experiments. Before the frozen sections of breast tissue were stained with Fluo-VIP™, they were stained with H&E staining to ascertain the presence of normal or cancerous tissue. The normal breast and breast cancer tissue sections appeared histologically very different from each other in the H&E-stained sections with mammary ducts being clearly visible in normal breast tissue (Figure 1B) and breast cancer showing numerous nests of cancer cells (Figure 1A).

Fluo-VIP™ homogeneously and highly stained the MNU-induced breast cancer tissue sections, as shown by homogeneous and intense fluorescence in Figure 1C. Only the nests of cancer cells were stained with fluorescence as revealed by the corresponding H&E-stained frozen sections. Staining was specific because the fluorescence was significantly reduced ($p < 0.05$, Table 1) in the presence of excess unlabeled VIP (Figure 1D) and PACAP (microphotographs not shown). The fluorescence intensity also reduced in the presence of excess secretin (microphotographs not shown), but to a slightly lesser extent than in the presence of excess VIP and PACAP.

Staining of normal breast tissues by Fluo-VIP™ (Figure 1E) was significantly less than that of breast cancer tissues ($p < 0.05$, Table 1). It was further reduced in the presence of excess unlabeled VIP (Figure 1F).

Discussion

This study documents the presence of VIP binding sites in MNU-induced breast cancer in rats, similar to breast cancer in humans. These sites are homogeneously distributed in the tissue sections as indicated by the even distribution of the fluorescence in the photographs of the breast cancer tissue sections. It is previously reported that VIP and PACAP both bind to

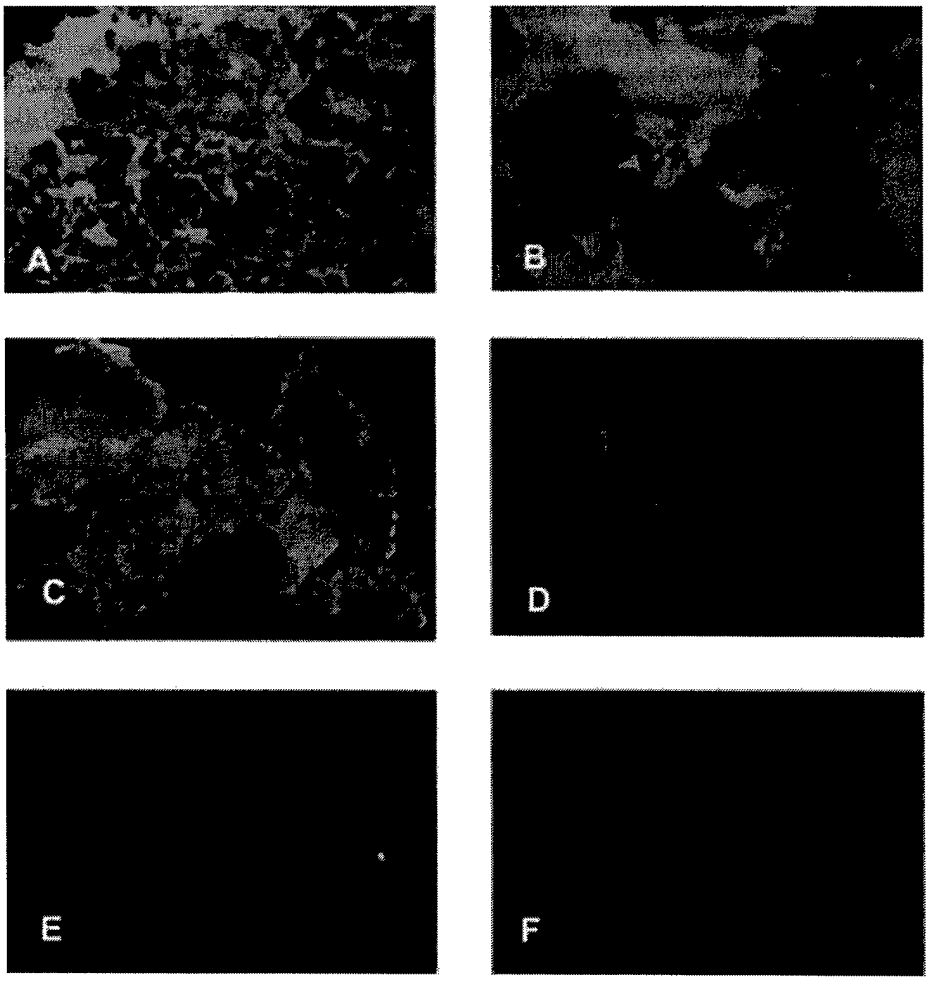

Figure 1. Microphotographs (40X) of hemotoxylin-eosin and Fluo-VIP^{TM} stained normal rat breast and MNU-induced rat breast cancer. (A) Hemotoxylin-eosin stained section of the MNU-induced rat breast tumor, showing numerous breast cancer cells. (B) Hemotoxylin-eosin stained section of the normal rat breast, showing normal mammary ducts. (C) Total staining of MNU-induced rat breast tumor with Fluo-VIP^{TM}, as indicated by intense fluorescence in the nests of cancer cells. (D) Non-specific staining of MNU-induced rat breast tumor with Fluo-VIP^{TM}, (in the presence of excess unlabeled VIP). Reduced fluorescence as compared to C indicates the specificity of staining of MNU-induced rat breast tumor with Fluo-VIP^{TM}. (E) Total staining of normal rat breast with Fluo-VIP^{TM}. Reduced fluorescence as compared to MNU-induced breast tumor (Figure 1C) indicates the presence of reduced number of VIP binding sites in normal breast in comparison to MNU-induced rat breast tumor. (F) Non-specific staining of normal rat breast with Fluo-VIP^{TM} (in the presence of excess unlabeled VIP).

VIP receptors (VPAC1 & VPAC2) with equal affinity [21]. The staining by Fluo-VIP^{TM} was specific as both unlabeled VIP and PACAP displaced the Fluo-VIP^{TM} from the tissue sections. In addition, the staining of normal rat breast tissues, was much less than rat breast cancer tissue, as shown by reduced fluorescence (Figures 1C & D), indicating the overexpression of VIP-R in MNU-induced rat breast cancer as compared to normal rat breast.

Of the two known types of VIP receptors, VPAC 1&2, secretin is shown to have significantly less affinity for VPAC2 receptors (IC_{50} 5,000–30,000 nM) as compared to VPAC1 receptors (IC_{50} 300 nM) [21]. Our results from this study agree with the previous findings, since Fluo-VIP^{TM} was displaced from the tissue sections by secretin indicating that most likely VPAC1 receptors are present in MNU-induced rat breast carcinoma. Similarly, others have shown that

VIP receptor of type VPAC1 is expressed in breast cancer as indicated by the presence of high levels of VPAC1 receptor mRNA in human breast cancer cells and biopsy tissues [13, 15]. Thus, the MNU-induced breast cancer is a suitable model to study human breast cancer with respect to the type of VIP-R expressed. However, more studies such as detection of mRNA of VIP-R subtype in the breast cancer tissue are needed to conclusively prove the type of VIP receptors expressed in this model.

Although, the presence of receptors can be detected using either tissue homogenates or histological sections, it has been shown that the histological sections are superior to the tissue homogenates to identify receptors [22]. The reason for this is that surgically resected tumor samples usually contain non-malignant tissue as well. The studies using homogenate tissue samples cannot reliably assess whether positive results are due to receptor expression in normal or malignant tissue, introducing a degree of bias into the results. Therefore, in this study we used histological tissue sections of normal and cancerous rat breast. By taking adjacent sections and staining with H&E, the presence of receptors in the malignant or normal tissues was clearly demonstrated.

Targeting of pharmaceuticals to breast cancer is useful for both diagnostic and/or functional imaging and delivery of therapeutics to the tumor. This could not only help in early detection and prognosis of breast cancer but also its effective chemotherapy. Passive targeting to tumors with the use of long circulating sterically stabilized liposomes (SSL) has been demonstrated. Due to leaky vasculature and lack of lymphatics in the tumor, long circulating SSL can extravasate at the tumor site and accumulate in the tumor [23]. However, high interstitial pressure in the tumor may inhibit retention of liposomes at the tumor site, hence active targeting by antibody–antigen or peptide–receptor binding interactions are required for high and homogenous accumulation of the liposomes in the tumor. Recently, SSL were shown to penetrate tumor mass more uniformly due to active targeting to breast cancer cell surface protein HER2 with the use of anti-HER2 Fab fragment [24]. We predict that breast cancer targeting will be even more effective when VIP-R is utilized. Our rationale for this are; first HER2 is expressed in only about 30% of the breast cancer, while VIP-R are almost always and homogeneously overexpressed in human breast cancer [11, 14]; and second, VIP is an endogenous peptide and has obvious advantages such as smaller size and no immune response, over antibodies such as anti-HER2. Previously we developed sterically stabilized liposomes that carry VIP on their surface [25, 26]. Our next goal is to use these liposomes to study active targeting of radionuclides and chemotherapeutic agents to breast cancer, using VIP as the targeting ligand and MNU-induced rat breast cancer as an animal model.

VIP regulates the growth and numerous functions in tumor cells. Therefore, the presence and absence of VIP-R may have prognostic implications. MNU-induced rat breast cancer is an established model used extensively to study mammary carcinogenesis. Based on the results of this study, it can now be used to investigate the role of VIP-R in tumorogenesis. Recently, some successes have been achieved in attempts to target the VIP-receptors using labeled VIP or its agonist for tumor localization [27, 28]. These studies were performed on nude mice bearing implanted cancer cells. These xenograft models do not have the environment in which the actual tumor exists. The MNU-induced *in situ* rat breast cancer model is superior to the xenograft model since it provides anatomically the right environment in which the actual tumor grows.

In conclusion, the results of this study indicate that MNU-induced breast cancer in rats overexpresses VIP receptors of type VPAC1, as compared to the normal breast. Active targeting to these receptors with the use of a VIP-coupled carrier can significantly improve the diagnosis, prognosis and therapy of breast cancer.

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VIP-LIPOSOMES FOR ACTIVE, CELL-SPECIFIC TARGETED DELIVERY TO BREAST CANCER IN VIVO

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ABSTRACT SUMMARY

Vasoactive intestinal peptide (VIP) receptors are overexpressed in breast cancer. This study investigated the targeting of labeled sterically stabilized liposomes with surface conjugated VIP to breast cancer in rats in vivo. VIP liposomes had significantly higher accumulation than liposomes without VIP indicating that passive and active targeting to breast cancer can be accomplished in vivo.

INTRODUCTION

Targeted delivery of radionuclides and therapeutic agents to tumors has important implications for detection, diagnosis and therapy of cancer. Biomarkers that differentiate cancerous tissue from normal tissues can be used as targets for this purpose. Since VIP receptors (VIP-R) are overexpressed in breast cancer (1), and these receptors do not express in the circulation they are promising targets. In order for VIP or its analog to interact with the VIP-R it needs to extravasate from the circulation. Particulate carriers such as liposomes with size of ~100nm can only extravasate at certain disease sites such as tumor or inflammation due to the presence of leaky vasculature. To this end, we aimed to develop a universal liposomal carrier system with active VIP on its surface for targeted delivery to breast cancer. In our previous studies we prepared sterically stabilized liposomes (SSL) encapsulating a radionuclide, technetium 99m-hexamethyl propylene amine oxide, (Tc99m-HMPAO) with non covalently associated VIP on their surface (2). When these liposomes were injected into breast tumor bearing rats and imaged by a gamma camera, no significant difference in image enhancement of the tumor was observed in the presence and absence of VIP (3). However, accumulation of liposomes in breast tissue with tumor was much higher than normal breast tissue (3). These results indicated that liposomes were passively targeted to breast cancer by extravasation but active targeting did not occur. We explained these results with the possibility of the VIP dissociating from liposomes at the receptor site. Recently we have developed a method to conjugate VIP on SSL covalently (4,5).

The purpose of this study was to prepare SSL encapsulating Tc99m-HMPAO with VIP covalently conjugated and test the tumor accumulation of these liposomes and compare it to SSL without VIP, in order to determine if any active targeting to breast tumor can be achieve by this novel carrier system.

EXPERIMENTAL METHODS

Preparation of Tc99m-HMPAO VIP-SSL

Tc99m-HMPAO VIP-SSL was prepared as previously described (5). Briefly, Tc99m-HMPAO SSL were first prepared by incubating lipophilic Tc99m-HMPAO complex with preformed glutathione-containing liposomes and separating the free label by gel filtration. Meanwhile VIP was conjugated to DSPE-PEG_{3400} as described before (5). DSPE-PEG_{3400}-VIP was incubated with Tc99m-HMPAO SSL at 37°C. The free DSPE-PEG_{3400}-VIP and the label were then removed by gel filtration to give Tc99m-HMPAO VIP-SSL. The size of both Tc99m-HMPAO SSL and Tc99m-HMPAO VIP-SSL was determined by quasi-elastic light scattering. The percent encapsulation of radiolabel was also determined for both SSL and VIP-SSL.

Breast tissue accumulation

Breast cancer was induced in female virgin rats with MNU as previously described (2,6).

Rats were anesthetized and were then given ~300 μCi of the assigned radiotracer (Tc99m-HMPAO encapsulating SSL or Tc99m-HMPAO encapsulating VIP-SSL) intravenously via the tail vein (minimum of 5 rats/group).

At 27 h post-injection, the rats were euthanized by overdose of ketamine/xylazine. Breast tissues (normal or tumor) were dissected out. They were washed with saline, dried between folds of paper towel and transferred to pre-weighed polypropylene tubes and capped. The tubes were then weighed and the weight of each of the tissue was determined. Their activity measured in a shielded well scintillation gamma counter. To correct for physical decay of Tc99m, and to permit calculation of the uptake of the radiolabeled liposomes, a 10μL aliquot of the injected dose was also counted. The results were expressed as percent injected dose per gram of tissue (% I.D. / g).

RESULTS AND DISCUSSION

The size and percent encapsulation of Tc99m-HMPAO SSL and Tc99m-HMPAO VIP-SSL were not significantly different from each other as shown in Table 1.

	Tc99m-HMPAO SSL	Tc99m-HMPAO VIP-SSL
SIZE, nm	109.81± 14.21	114.77± 13.72
LABELING EFFICIENCY	85.7 ± 4.46 %	83.8 ± 2.42 %

Table 1. Characteristics of Tc99m-HMPAO encapsulating SSL and VIP-SSL (each value, mean ± standard deviation, n = 6)

Both Tc99m-HMPAO SSL and Tc99m-HMPAO VIP-SSL showed significantly higher accumulation in breast tumor as compared to normal breast tissue, suggesting passive targeting of both liposomes to the tumor occurred most probably due to extravasation of the liposomes through leaky vasculature of the tumor (figure 1). However, the Tc99m-HMPAO SSL with VIP showed significantly higher accumulation in breast tumor as compared to liposomes without VIP. This demonstrates that active targeting of liposomes to breast cancer has been achieved by VIP and VIP-R (Figure 1).

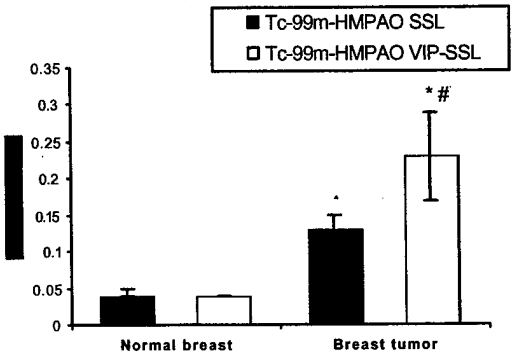

Figure 1. Accumulation of Tc-99m-HMPAO encapsulating SSL and Tc-99m-HMPAO encapsulating VIP-SSL in normal breast tissue and breast cancer (Each data, n=5, mean ± SEM).

* $p<0.05$ compared to Tc-99m-HMPAO encapsulating SSL in breast tumor

\# $p<0.05$ compared to Tc-99m-HMPAO encapsulating SSL and VIP-SSL in normal breast

^ $p<0.05$ compared to Tc-99m-HMPAO encapsulating SSL and VIP-SSL in normal breast

CONCLUSIONS

The results of this study indicate that the covalent conjugation of VIP on the surface of Tc99m-HMPAO SSL did not alter the characteristics of the liposomes, such as size and percent encapsulation. However, breast tumor accumulation of the Tc99m-HMPAO SSL was significantly higher in the presence of VIP due to active targeting. Hence, we have developed a novel carrier system, which is both passively and actively targeted to breast cancer. We are currently evaluating this targeted carrier system for the enhanced

imaging and effective therapy of breast cancer.

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